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Folate Reductase of the Amethopterin-Resistant *Streptococcus faecium* var. *durans*/A_k

I. Inhibition by Amethopterin and Methasquin, a New Quinazoline Antifolate

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SUMMARY

The amethopterin-resistant bacterium *Streptococcus faecium* var. *durans*/A_k (SF/A_k) synthesizes at least two species of tetrahydrofolate dehydrogenase. The more abundant species, designated *folate reductase*, catalyzes the pyridine nucleotide-dependent reductions of folate and dihydrofolate. It has been purified 68-fold and resolved from a minor species. The minor species of enzyme apparently is the *specific dihydrofolate reductase*, which is prevalent in the amethopterin-sensitive parent strain, *S. faecium* var. *durans*/O (SF/O), and mediates only dihydrofolate reduction.

Folate reductase has an estimated molecular weight of 22,500. Like the tetrahydrofolate dehydrogenases of animal sources, it catalyzes dihydrofolate reduction more rapidly than folate reduction. Several kinetic characteristics of folate and dihydrofolate reduction were determined. Similarities between the folate reductase of SF/A_k and the nonbacterial tetrahydrofolate dehydrogenases pointed to the usefulness of this bacterial folate reductase in evaluating the reductase-inhibitory activity of new chemicals synthesized as potential chemotherapeutic agents. Analyses of the inhibition of folate and dihydrofolate reduction by amethopterin and by a new quinazoline antifolate, *N*-[*p*-[[(2,4-diamino-5-methyl-6-quinazolinyl)methyl]amino]benzoyl]-L-aspartic acid, designated methasquin, suggested that the latter may be beneficial clinically and, perhaps, more effective than amethopterin. The enzyme-methasquin interaction is slightly firmer than the enzyme-amethopterin interaction and, unlike the latter, is relatively independent of experimental conditions.

INTRODUCTION

The enzymatic synthesis of tetrahydrofolic acid is mediated by the enzyme(s) 5,6,7,8-tetrahydrofolate:NAD^+ ($NADP^+$) oxidoreductase (EC 1.5.1.3), called tetrahydrofolate dehydrogenase, folate (folic) reductase, and, frequently, dihydrofolate (-ic) reductase. Tetrahydrofolate dehydrogenases isolated from neoplastic sources (1–3) catalyze the reduction of folic and dihydrofolic acids. Zakrzewski *et al.* (2) named the purified reductase of the amethopterin-resistant subline of cultured Sarcoma 180 cells "folate reductase," although the enzyme mediates the reduction of folate more slowly than that of dihydrofolate. Perhaps this designation is physiologically more appropriate than "dihydrofolate reductase," since there are reductases, such as those isolated from *Streptococcus faecium* var. *durans*

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ATCC 8043 (4), from the folate-synthesizing bacteria (5, 6), and from protozoa (7), that display activity with dihydrofolate, but not with folate.

The folate-dependent, amethopterin-sensitive bacterial strain *S. faecium* var. *durans*/O (SF/O) synthesizes two types of tetrahydrofolate dehydrogenase: *folate reductase*, which mediates the reduction of both folate and dihydrofolate, and a *specific dihydrofolate reductase*, the predominant enzyme, which fails to reduce folate (8). Both contribute to the total dihydrofolate reductase activity of SF/O. In the amethopterin-resistant mutant strain *S. faecium* var. *durans*/A_k (SF/A_k), folate reductase is the major form of tetrahydrofolate dehydrogenase. Exponentially growing cells of SF/A_k synthesize at least 200 times more folate reductase than SF/O. Presumably, the drug resistance of SF/A_k depends upon the increased synthesis of folate reductase, because the level of specific dihydrofolate reductase in exponentially growing cells of SF/A_k is markedly lower than in SF/O.

Interest in the modes of resistance to cancer chemotherapeutic drugs prompted the study of the folate reductase of SF/A_k. Apparent resemblance, in general, to the enzyme of animal cells and, specifically, to the enzyme of neoplastic cells with respect to folate reduction suggested that the folate reductase of SF/A_k might be the preferable form of tetrahydrofolate dehydrogenase for use in evaluating reductase inhibitors as potential chemotherapeutic agents.

This paper describes the partial purification and several properties of the folate reductase of SF/A_k as well as the characteristics of folate and dihydrofolate reduction. It also deals with the inhibition of folate and dihydrofolate reduction by amethopterin and by methasquin, a 2,4-diaminoquinazoline antifolate (9) selected for clinical trial. The influence of intracellular environment on the interaction of enzyme and inhibitor is considered.

MATERIALS AND METHODS

Materials

Chemicals. The source and/or preparation of most of the chemicals used in these studies were described previously (8, 10).

Amethopterin (a gift from Dr. Harriet Kiltie, American Cyanamid Company) was purified by ion-exchange chromatography. The fractions used were determined to be free of inhibitory contaminants by bioautographic analyses as described previously (10). The concentration of solutions of amethopterin was based on the extinction coefficient in 0.1 N NaOH of 23,000 M^{-1} cm^{-1} at 257 nm (11). Methasquin, the quinazoline antifolate *N*-[*p*-[[(2,4-diamino-5-methyl-6-quinazolinyl)methyl]amino]benzoyl)-L-aspartic acid disodium salt pentahydrate, was synthesized by Dr. John Davoll (Parke, Davis and Company, Middlesex, England). Dr. Davoll, Dr. John Dice (Parke, Davis and Company, Ann Arbor, Mich.), and Dr. Harry B. Wood, Jr. (Cancer Chemotherapy National Service Center, Bethesda, Md.), supplied bioautographically pure (9) samples of this compound. DEAE-cellulose, purchased from Mann Research Laboratories, was washed sequentially with 0.3 N HCl, water until neutral, 0.5 N KOH, and water until neutral (12). It was stored in glass-distilled water at 4°. Prior to use, it was equilibrated with standard buffer (10 mM potassium phosphate, pH 7.4, 1 mM with respect to EDTA). Reference proteins, bovine albumin (mol wt 67,000), ovalbumin (45,000), chymotrypsinogen A (25,000), and myoglobin (17,800) were obtained from Mann Research Laboratories; and ribonuclease (13,700), from the Worthington Biochemical Corporation.

Source of folate reductase. SF/A_k is a mutant strain of SF/O. In a purine- or pyrimidine-free medium (13), SF/A_k is resistant to 2000–3000-fold higher concentrations of amethopterin. An extract of late exponential SF/A_k cells obtained by continuous cultivation (14) was prepared and treated according to published methods with protamine sulfate (15) and ammonium sulfate (10). The ammonium sulfate fraction, identified as Fraction 1R (10), was then treated as described below.

Methods

Enzyme purification. Consecutive molecular sieve and ion-exchange chromatography was employed to increase the purity of Fraction 1R. An 8-ml portion (117.6 mg of pro-

tein, 67.4 dihydrofolate reductase units) was used for each filtration through a 3 × 47 cm column of Sephadex G-100; standard buffer was the eluent (Fig. 1). Resultant fractions (6.9 ml), which had specific activity values of 4–14 dihydrofolate reductase units/mg of protein, were combined; a 33-ml portion of the combined material was used for each column (1 × 35 cm) of DEAE-cellulose (Fig. 2). After adsorption on the column and elution with 40 ml of standard buffer, elution with a gradient of increasing concentration of NaCl in standard buffer was initiated. The gradient was achieved by flowing 1.2 M NaCl–standard buffer solution into standard buffer in the constant-volume (800-ml) mixing chamber. Immediately after elution from the DEAE-cellulose, the refractive indices of the fractions (6.9 ml) were read. Fractions were dispensed into sacs of dialysis tubing and concentrated with solid sucrose (8). After 18–20 hr under sucrose at 4°, the dialysis sacs were rinsed free of adhering sucrose and immersed in 20 ml of cold standard buffer for 10 min, and each treated fraction was transferred to a Pyrex storage tube.

Determination of enzyme activity. The spectrophotometric method (8) for measuring dihydrofolate reductase activity as decrease in absorbance at 340 nm at pH 6.5 and 30° was used to follow the course of the purification. One unit of dihydrofolate reductase activity is the amount required to catalyze the reduction of 1 μmole of dihydrofolate per minute under the assay conditions described (8). The assay of folate reductase activity (8) was based on the method of Raunio and Hakala (16). To initiate the reaction, NADPH (0.08 mM) was added to the other components of the reduction mixture (0.22 mM folate, 10 mM $NaHCO_3$, and enzyme preparation) in 0.1 M sodium citrate, pH 5.9. After incubation at 37°, the reduction product, presumably tetrahydrofolate, was cleaved by acid, and the resultant diazotizable amine was determined by absorbance at 560 nm. One unit of folate reductase activity is the amount required to catalyze the formation of 1 μmole of tetrahydrofolate per minute.

Inhibition studies. The general procedure for the kinetic analysis of inhibition has been described (10). Standard enzyme assay conditions were used, except that the inhibitors were incubated with the enzyme for 5 min at 30° prior to dihydrofolate reduction, or for 10 min at 25° prior to folate reduction, under the specific experimental conditions described in the legends to Figs. 9 and 10.

Estimation of molecular weight. Calculations were based on the filtration behavior of folate reductase and the reference proteins on a column (2.5 × 87 cm) of Sephadex G-100 equilibrated with standard buffer at 4°. The method of Whitaker was followed (17).

Electrophoretic analyses. The conditions and details of zone electrophoresis with starch gel and visualization of enzyme bands and protein were described previously (8).

RESULTS

Purification. The dihydrofolate reductase assay was used to follow the course of purification for reasons of economy. Dextran gel filtration separated reductase activity from the bulk of protein (10) and effected a substantial purification of the dihydrofolate reductase activity of SF/A_k (Fig. 1). Ion-exchange chromatography with DEAE-cellulose (Fig. 2) improved the purification and also resolved the major form of reductase from a minor, more electronegative form (Figs. 2 and 3). The most highly purified preparations of the major form were the fractions usually eluted by 0.08–0.1 M NaCl. As summarized in Table 1, the activity of peak eluates represented 68-fold purification with 24% recovery. Electrophoretic analyses of ion-exchange fractions

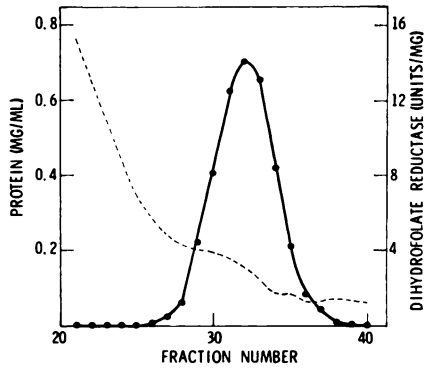

FIG. 1. *Purification of folate reductase of SF/A_k by molecular sieve chromatography*
Details are presented in METHODS.

(Fig. 3) substantiated the presence of a distinctly different species of dihydrofolate reductase in fractions comprising the minor peak eluted by 0.15–0.2 M NaCl.

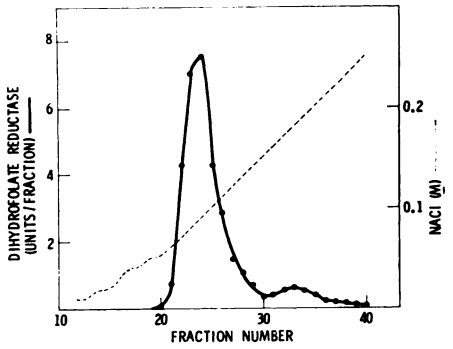

FIG. 2. *Elution of folate reductase of SF/A_k from DEAE-cellulose with sodium chloride*
Details are described in METHODS.

Lability and stabilization of enzyme activity. Sucrose was used to concentrate (7-fold) the NaCl eluates and to stabilize the enzyme activity. Highly purified preparations in dilute solution that had been frozen at −20° after elution from DEAE-cellulose and then thawed lost 30–50% of their activity. Storage in 0.23 mM NADPH had no beneficial effect. When stored at −20° after sucrose concentration, preparations retained activity for as long as 2 years. After concentrated preparations had been diluted in standard buffer, the activity was lost within several minutes. A diluent of standard buffer containing 0.05% bovine albumin maintained the activity of the enzyme during experimentation. Thus, bovine albumin at a final concentration of 0.003% was included in all experimental reaction mixtures.

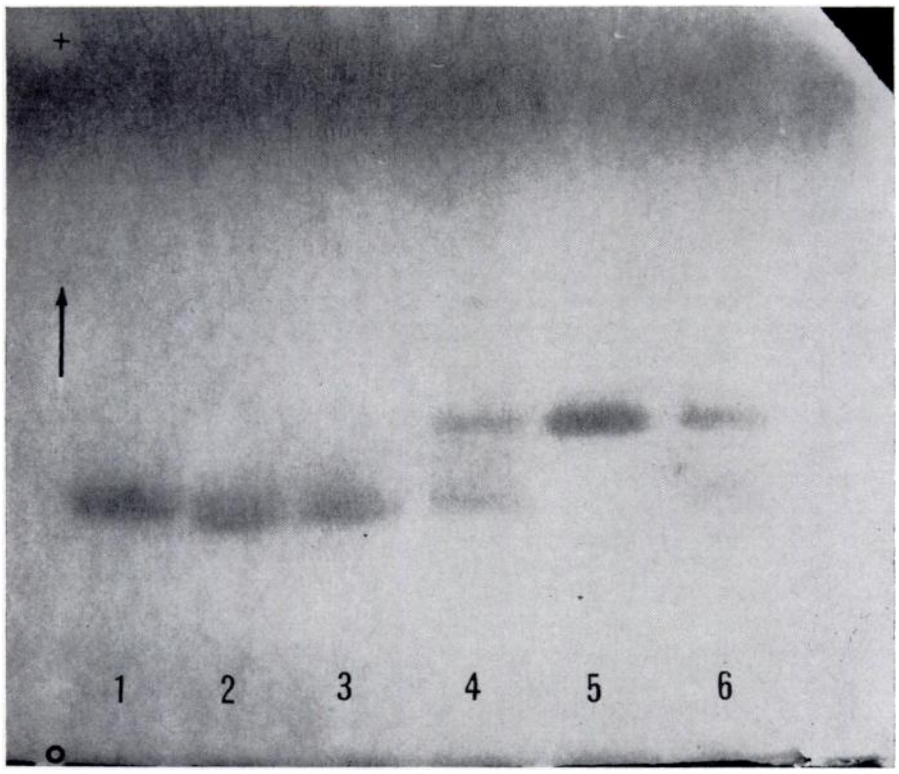

FIG. 3. *Zone electrophoresis in starch gel of dihydrofolate reductases of SF/A_k*
Experimental details of electrophoresis and visualization of enzyme activity bands were described previously (8); O represents the origin, and +, the anodal region. Portions of DEAE-cellulose fractions were analyzed in the following order: slots 1 and 2, major elution peak fractions (0.05 unit of dihydrofolate reductase activity per slot); slot 3, late fraction of descending limb of major peak (0.06 unit); slot 4, pooled fractions of trailing end of major peak (0.04 unit); slot 5, pooled minor peak fractions (0.02 unit); slot 6, combination of major peak (0.025 unit) and minor peak (0.01 unit) fractions.

TABLE 1

Purification of folate reductase from S. faecium var. durans/A_k

Fraction	Volume	Total protein[a]	Dihydrofolate reductase activity		
			Specific activity	Total activity[a]	Recovery[a]
	ml	*mg*	*units/mg*	*units*	%
Cell extract	85.0	1870.0	0.43	802	100
Fraction 1R	61.6	905.5	0.57	519	65
Combined Sephadex G-100 filtrates	323.8	45.3	10.18	463	58
DEAE-cellulose eluates					
Major peak (0.07–0.09 M NaCl)	30.2	6.5	29.3	190	24
Intermediate fractions (0.10–0.14 M NaCl)	50.4	6.7	15.7	105	13
Minor peak (0.15–0.20 M NaCl)	60.4	5.8	4.9	28	3.5

[a] Values were computed on the basis of 85 ml of bacterial extract.

Molecular weight. The molecular weight of the folate reductase of SF/A_k was estimated to be 22,500 (Fig. 4).

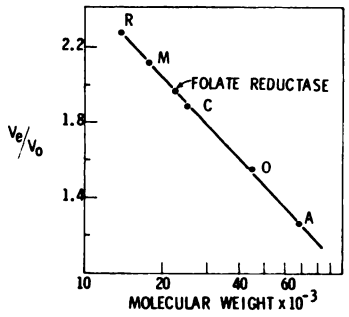

FIG. 4. *Semilogarithmic plot of V_e/V_o with respect to molecular weight of protein markers and folate reductase*

The void volume (V_o) was determined by elution of blue dextran (absorbance at 625 nm). The absorbance of proteins was determined at 280 nm except for myoglobin, which was read at 415 nm. The volume at peak elution of the proteins (*A*, bovine albumin; *O*, ovalbumin; *C*, chymotrypsinogen A; *M*, myoglobin; *R*, ribonuclease) was regarded as V_e.

Reduction of Dihydrofolate

Effect of ions. KCl (0.1 M) was a constituent of the standard dihydrofolate reductase reaction mixture, in 50 mM potassium phosphate buffer, pH 6.5. Either KCl or NaCl at concentrations of 0.10–0.33 M in the standard reaction mixture stimulated the dihydrofolate reductase activity of the folate reductase by 38%. LiCl (0.10–0.33 M) and NH_4Cl (1 M) increased activity by 50%. $MgCl_2$ between concentrations of 0.02 and 0.1 M was slightly stimulatory (17%), but at higher concentrations it was inhibitory. The chlorides of Ba^{++}, Ca^{++}, Co^{++}, Cu^{++}, and Mn^{++} could not be tested because of precipitation in the reaction mixture or interference with absorbance at 340 nm. Sodium acetate (0.1–0.7 M) increased activity by 26%; sodium citrate at these concentrations had no activating effect and at 1 M was inhibitory.

Effect of KCl on pH optimum for dihydrofolate reduction. The reduction of dihydrofolate under conditions of salt activation was studied in 50 mM potassium phosphate buffer in the pH range of 5.9–7.9. Reaction rates were most rapid between pH 6.3 and 6.7, the range also optimum in the absence of KCl (10). Thus, pH 6.5 was selected for kinetic studies.

Effect of KCl on dihydrofolate reduction with varied concentrations of reactants. When the concentration of the substrate under study was varied under otherwise standard reaction conditions, the addition of KCl increased the initial reaction velocity with concentrations of 1.7–50 μM NADPH and 2–34 μM dihydrofolate. The interaction of NADPH with enzyme reflected Michaelis-Menten kinetics in both the presence and absence of KCl (Fig. 5). A double-reciprocal plot (18) of the data (Fig. 5B) illustrates that the salt increased V_{max} and slightly decreased the K_m value. From these and similar results, the K_m values for NADPH were calculated to be 4.22 μM (in the absence of KCl) and 3.33 μM (in the presence of KCl). The interaction of dihydrofolate with the enzyme, as illustrated in Fig. 6, differed. Two slopes characterized the double-reciprocal plots of the data for the control and salt-stimulated reactions (Fig. 6B), which suggested an unusual interaction of dihydrofolate and the enzyme. KCl had little, if any,

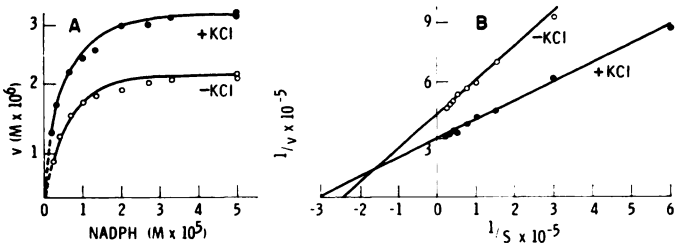

FIG. 5. *Effect of concentration of NADPH on initial velocity of dihydrofolate reduction in the absence* (○) *and presence* (●) *of 0.1 M KCl*

Initial velocity (v) is expressed as moles per liter of dihydrofolate reduced per minute.

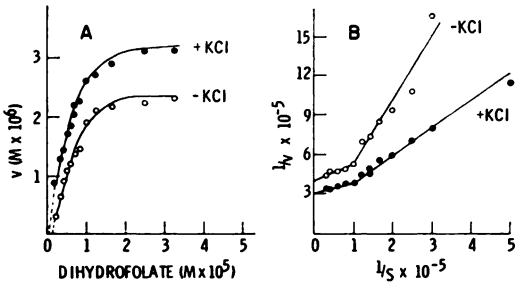

FIG. 6. *Effect of concentration of dihydrofolate on initial velocity of dihydrofolate reduction in the absence* (○) *and presence* (●) *of 0.1 M KCl*

Initial velocity (v) is expressed as moles per liter of dihydrofolate reduced per minute.

effect on the concentration of dihydrofolate required for half-maximal velocity. The K_m values for dihydrofolate determined from the plots of v with respect to S (Fig. 6A) were 4.5 μM in the presence and 5.5 μM in the absence of KCl.

Inhibition by folate and NADP. Folate (7.6 μM) produced competitive inhibition when incubated for 5 min at 30° with the purified reductase and NADPH prior to the addition of various amounts of dihydrofolate to a series of reaction mixtures. Folate decreased by 50% the initial reaction velocities observed with 12.5 μM and lower concentrations of dihydrofolate; the presence of 16.7 μM dihydrofolate reversed the inhibition. Tetrahydrofolate, assumed to be practically free of dihydrofolate on the basis of absorbance maximum at 297 nm and negligible absorbance at 340 nm, inhibited the reaction poorly. In the presence of 0.13 mM tetrahydrofolate, the activity was 67% of the control value. NADP at 30 μM inhibited the velocity of each of a series of reactions containing 7–50 μM NADPH. NADP, which was competitive with NADPH, increased the K_m value for NADPH 3-fold. NAD at 10 μM was not inhibitory.

Reduction of Folate

Whereas the reduction of dihydrofolate was studied directly by following the decrease in absorbance at 340 nm during the NADPH-dependent reduction, a coupled assay procedure (8, 16) was used to study the reduction of folate. Step 1 involved the enzymatic reduction of folate, and step 2, the measurement of the enzymatic product after acid cleavage to diazotizable amine (absorbance at 560 nm). The coupled assay procedure was more economical than a spectrophotometric determination similar to the dihydrofolate reductase assay. With the standard enzymatic reaction mixture (step 1), a linear relationship was noted between the extent of the reaction catalyzed during 30 min and the amounts of added cell extract up to an absorbance of the resultant diazotized amine approaching 0.4. The velocity of step 1 was also linearly proportional to the duration of incubation until the enzymatic product reached an amount equivalent to diazotized amine with absorbance of 0.4. The velocity of the reaction catalyzed by highly purified preparations was linearly proportional to enzyme concentration (20 min of incubation) and time from 0 to 20 min.

The abundance of this enzyme in SF/A_k cells minimized the interference of nonspecific cell constituents in the assay of folate

reductase activity. By contrast, as previously pointed out (8), the relatively greater presence of interfering factors in SF/O hindered measurement of the meager amounts of folate reductase activity in extracts of this strain. Under certain conditions of assay (8), acceptable estimations of activity in SF/O extracts could be made.

Effect of pH and KCl. The buffer for the standard folate reductase reaction mixture was 100 mM sodium citrate (pH 5.9), 10 mM in sodium bicarbonate; KCl was not included. Tests showed that 50–300 mM KCl did not stimulate or inhibit the enzyme activity in the citrate buffer system. The optimum range for folate reduction in citrate was pH 5.4–5.75; at pH 5.9, the reaction was 10% lower. The increased stability of NADPH at pH 5.9 prompted the selection of 5.9 as the standard pH of the system. In a buffer of phosphate and citrate, activity was optimum around pH 5.0; 100 mM KCl decreased the activity by 10% when the pH range was 4.7–6.0 but it had no influence when the range was 6.4–7.0.

Reactants. With folate as substrate, the enzyme was specific for NADPH as the reductant, and NADH (0.08 mM) could not replace NADPH. The reaction with 0.08 mM NADPH was not inhibited by 0.03–0.24 mM NADH. When tested separately in the standard reaction, 0.24 mM NAD and 0.24 mM NADP decreased the activity by 11–15%.

The effect of folate and NADPH concentration on the velocity of folate reduction was investigated by using Sephadex G-100 filtration fractions. Results with more highly purified enzyme preparations were similar. The reaction conditions were standard except that the concentration of the substrate under study was varied and the initial reaction velocity was based on 10 min of incubation at 37°. The initial reaction velocity–NADPH concentration relationship followed the Michaelis-Menten equation (Fig. 7A). An apparent K_m value of 14.3 μM NADPH was derived from a double-reciprocal plot of the data (Fig. 7B). Studies with supplemental KCl yielded similar data and K_m value.

A graphical representation of the interaction of folate with the enzyme also reflected Michaelis-Menten kinetics (Fig. 8). Velocity was half-maximal at 29 μM folate under standard conditions modified as indicated above. With KCl in the system, 30 μM folate was the K_m value.

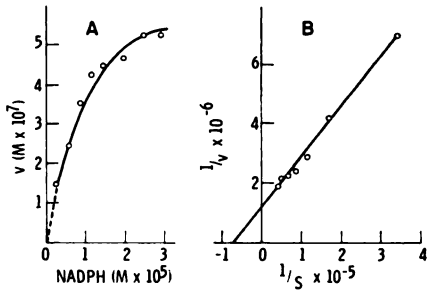

FIG. 7. *Effect of concentration of NADPH on initial velocity of folate reduction*

Initial velocity (v) is expressed as moles per liter of tetrahydrofolate formed per minute.

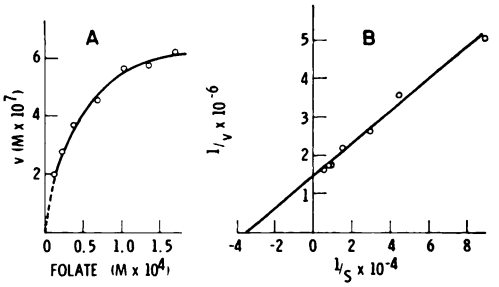

FIG. 8. *Effect of concentration of folate on initial velocity of folate reduction*

Initial velocity (v) is expressed as moles per liter of tetrahydrofolate formed per minute.

Inhibition of Enzyme Activity

Inhibition by amethopterin and methasquin (9) was investigated under several different conditions (Figs. 9 and 10) with DEAE-cellulose fractions.

Inhibition of dihydrofolate reduction in the presence of KCl. Varied amounts of amethopterin, each less than 25 nmoles/mg of total protein in the enzyme preparation, inhibited the reduction linearly. However, the conditions under which the enzyme was incubated with inhibitor at pH 6.5 and 30° prior to the reaction affected the extent of the resultant inhibition (Fig. 9A). Simultaneous prior incubation of enzyme with amethop-

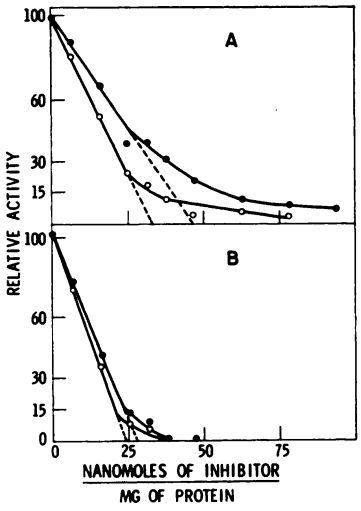

FIG. 9. *Inhibition of dihydrofolate reduction by amethopterin (A) and methasquin (B) in the presence of KCl*

Enzyme activity was measured after incubation of the enzyme preparation with varied amounts of the inhibitors in the absence (condition 1, ●) or presence of 0.1 mM NADPH (condition 2, ○). Reduction was initiated by the addition to the previously incubated mixtures of 0.1 mM NADPH and 33 μM dihydrofolate (condition 1) or of 33 μM dihydrofolate alone (condition 2). Reductase activity is expressed as activity relative to the reduction measured in the absence of inhibitor, taken as 100. Inhibitor quantities are described on the basis of 1 mg of SF/A_k total protein; 0.11 μg of SF/A_k protein per milliliter was the experimental amount (specific activity, 20 units).

terin and NADPH (condition 2) produced more efficient inhibition. Relaxed inhibition occurred with the late addition of NADPH (condition 1). Thus, when the inhibitor to enzyme ratio (I/E) was 25 nmoles/mg of total protein, condition 1 effected only 50% inhibition and condition 2 gave 75% inhibition. Titration with methasquin yielded different results (Fig. 9B). Generally, inhibition by amounts of methasquin equimolar to amethopterin was stronger and was influenced only weakly by prior incubation conditions. The quinazoline inhibited proportionally at I/E ratios less than 22 nmoles/mg of total protein. With an I/E ratio of 22 nmoles/mg, the reduction was inhibited by 80% under condition 1 and by 88% under condition 2. The differences in degree of inhibition produced by the two compounds under each set of conditions were emphasized by the titration values obtained by extrapolating the linear portion of each plot to the abscissa. (Titration values express the amount of drug that would completely inhibit the enzyme in 1 mg of total protein in the preparation if inhibition were linear to zero activity.) For amethopterin (Fig. 9A), 46.5 nmoles (condition 1) and 32.5 nmoles (condition 2), and for methasquin (Fig. 9B), 27 nmoles (condition 1) and 24 nmoles (condition 2) were the extrapolated amounts.

Inhibition of dihydrofolate reduction in the absence of KCl. Plots of data obtained by titration with amethopterin and methasquin in the absence of KCl showed that inhibition after simultaneous preliminary incubation of enzyme with inhibitor and NADPH was linearly proportional over a larger range of I/E ratios than inhibition following prior incubation without NADPH. Like inhibition in the presence of KCl, inhibition with the higher concentration range of amethopterin was somewhat more relaxed by the absence of NADPH during preliminary incubation. The titration values for both drugs obtained by extrapolating the linear portions of the plots to the abscissas, however, differed little (10–15%) from the value determined for methasquin after simultaneous incubation with enzyme plus NADPH.

Inhibition of folate reduction. At pH 5.9, also, simultaneous preliminary incubation of enzyme with amethopterin and NADPH strengthened the inhibitory activity of amethopterin. The amount of inhibition by methasquin, negligibly affected by prior incubation conditions, was greater than the inhibition by amethopterin. The addition of KCl to the folate reductase system did not modify the general characteristics of inhibition, but did increase the effectiveness of both compounds. The data for the inhibition of folate reduction in the presence of KCl are illustrated in Fig. 10; in general, these plots resemble those describing the inhibition of dihydrofolate reduction (Fig. 9). However, the values derived by titrating the enzyme under conditions of folate reduction were lower than those obtained from the data plotted in Fig. 9.

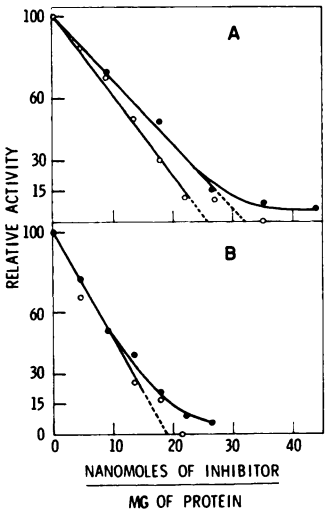

FIG. 10. *Inhibition of folate reduction by amethopterin (A) and methasquin (B) in the presence of 0.1 M KCl*

Enzyme activity was measured after incubation of varied amounts of inhibitors and folate reductase in the absence (condition 1, ●) and presence (condition 2, ○) of 0.08 mM NADPH. Reduction was initiated by the addition to the previously incubated mixtures of 0.08 mM NADPH and 0.22 mM folate (condition 1) or of 0.22 mM folate alone (condition 2). Reaction mixtures contained 1.14 μg of protein of a folate reductase preparation with specific activity values of 0.23 folate reductase and 20 dihydrofolate reductase units. Inhibitor quantities are described on the basis of 1 mg of total protein.

DISCUSSION

SF/A_k is a rich bacterial source of a tetrahydrofolate dehydrogenase which differs distinctively from most bacterial dihydrofolate reductases. A fundamental characteristic which distinguishes this tetrahydrofolate dehydrogenase, the folate reductase of SF/A_k, is its catalysis of the reduction of folate as well as dihydrofolate. For SF/A_k, as for all bacterial strains requiring supplemental folate for growth, synthesis of a folate reductase is a paramount function. The isolation from *Lactobacillus leichmannii* of an enzyme which reduces folate and dihydrofolate (19) also substantiates the necessity of this form of reductase in folate-dependent bacteria. In this respect, folate auxotrophs resemble animal cells. By contrast, folate reduction is circumvented in folate-independent bacteria, since dihydrofolate is the primary product in the biogenesis of folate coenzymes (20); for such bacterial systems, a specific dihydrofolate reductase is adequate. It is suggested that the properties of the folate reductase of SF/A_k are predominantly responsible for the resistance to amethopterin, because in the drug-sensitive SF/O cells only 10% of the total dihydrofolate reductase activity is attributable to folate reductase (8).

Also present in exponentially growing cultures of SF/A_k is an extremely low level of an enzyme which is indistinguishable from the specific dihydrofolate reductase of SF/O (8, 10) in electrophoretic mobility (Fig. 3), turnover of dihydrofolate, and gel filtration behavior.[1] The role of this minor reductase in tetrahydrofolate synthesis should not be minimized (8).

The bacterial folate reductase resembles the dihydrofolate reductase of lymphoma (21, 22) and Ehrlich ascites carcinoma cells (1) in the ion-dependent activation of dihydrofolate reduction. KCl activated the mammalian enzyme 3–5-fold, but increased the activity of the bacterial enzyme by only 40%. Activation of the bacterial enzyme probably also depends upon alteration of the catalytic site, which increases the turnover of dihydrofolate, as explained by Perkins and Bertino (21) and Reyes and Huennekens (22). A dissimilarity which influences the pH optima may contribute to the extent of activation. The pH optimum of the bacterial reductase was not altered by KCl; however, a shift from double pH optima to a single pH optimum after the addition of KCl has been observed with mammalian enzymes (1, 3).

Several kinetic characteristics of dihydrofolate reduction catalyzed by the folate reductase of SF/A_k parallel those described for animal systems (1–3, 12, 22). A calculated turnover of 816 moles of dihydrofolate per mole of folate reductase approaches values determined for the enzyme of neoplastic cells (1–3). The K_m value of 3.3 μM NADPH agrees with the kinetic constant determined for several mammalian

[1] A. M. Albrecht, unpublished observations.

reductases (1, 3, 12). A similar value was reported for the enzyme of the drug-sensitive Lymphoma L1210 in the absence of KCl (22), although KCl increased the K_m for the lymphoma enzyme 4-fold.

The unusual interaction of the folate reductase with dihydrofolate is not understood. Similarly abnormal behavior has been reported for the avian liver enzyme (12). The curve for the data obtained in the absence of KCl intersects the abscissa to the right of the zero intercept of the coordinates and appears sigmoidal. Sigmoid plots of initial velocity data typify many "allosteric enzymes" (23, 24), of which many have subunit structure (25). The small molecular weight of folate reductase (22,500), however, militates against a hypothesis involving subunit structure. It is possible that the velocity data for the low concentrations of substrate reflect the limitations of the spectrophotometric assay. A more sensitive method of kinetic analyses may help to clarify the basis of the interaction of dihydrofolate with folate reductase.

Apparently, folate and dihydrofolate are reduced via different mechanisms. In these experiments folate was reduced optimally within the more acid pH range of 5.4–5.75, and more slowly than dihydrofolate. A turnover number of 12.1 was calculated for folate reduction. KCl had no stimulatory effect. The kinetic constants were significantly higher for folate reduction: the K_m value for NADPH (14.3 μM) was 4 times greater, and the K_m for folate (30 μM), 6 times greater, than the corresponding value for dihydrofolate.

Whether the differences between the inhibition of folate reduction and dihydrofolate reduction reflect dissimilar mechanisms of inhibition is doubtful. Inhibition by amethopterin is extremely dependent on pH (26–28) and, like inhibition by aminopterin (4), results from a mutual binding of enzyme with inhibitor which is dependent upon appropriate incubation conditions. At pH 5.9, inhibition of the reduction of folate resembled the stoichiometric inhibition noted for the mammalian folate reductase (29). It is possible that the inhibition of dihydrofolate reduction reflected a slight dissociative effect at pH 6.5 on the inhibitor-enzyme complex (26–28), and perhaps also depended on the lower temperature used for dihydrofolate reduction. The involvement of NADPH in the folate reductase–amethopterin complex, especially at pH 6.5 in the presence of KCl, is consonant with the hypothesis that a firm ternary complex (30) is responsible for the strongest inhibition by amethopterin.

These observations, which corroborate the findings of others, emphasize the effects of experimental conditions upon enzyme-inhibitor binding. As a measure of reductase concentration in cell extracts, in lysates, or in purified enzyme preparations, titration with amethopterin (29) is a valuable technique but susceptible to modification by many factors. The method has been based on the stoichiometric inhibition of mammalian folate reductase by the 4-aminofolate analogues at pH 6.1 and 37° originally described by Werkheiser (29). Since the incubation mixture used by Werkheiser contained an NADPH-regenerating system, it may be presumed that NADPH was present during the exposure of enzyme to the 4-aminofolate analogues. Obviously, the conditions used by Werkheiser were optimum for the stoichiometric binding of reductase, upon which determinations of enzyme concentration could be based. The titration plots in Figs. 9 and 10 suggest that any small deviation from optimum conditions, whether involving pH, the temperature of incubation, the action of the reductant, or the salt concentration, may influence the reductase-amethopterin interaction and yield pseudostoichiometric binding and different titration values. Apparently, quantitation of reductase on the basis of titration with amethopterin is restricted to a precise set of conditions.

The quinazoline is not very much better than amethopterin as an inhibitor of tetrahydrofolate formation under the conditions studied. Nevertheless, it is evident that the inhibition by methasquin reflects a firmer binding to the enzyme that is relatively independent of simultaneous interaction with NADPH.

It has been assumed that 1 molecule of amethopterin combines with one active center of enzyme and that one active center exists per enzyme molecule (26). If the data

concerning inhibition by amethopterin and methasquin reflect only interaction at the active center, one may conclude that the firmest molecular interaction of inhibitor and enzyme at the active center causes the most pronounced inhibition. In such a case, methasquin may be considered the better titrating reagent. (The NADPH independence of inhibition by methasquin does not negate the binding of methasquin at the active site).

The molar concentration of the purified preparation used in the inhibition analyses was calculated on the assumption that the inhibition data resulting from the titration of folate reductase activity with methasquin at 37° and pH 5.9 (Fig. 10B) did indeed manifest the firmest equimolecular interaction of inhibitor with enzyme. On the basis of a molecular weight of 22,500, 1 mg of pure reductase protein would be equivalent to 44.4 nmoles of enzyme. The extrapolated titration point was 19 nmoles, indicating 43% purity. Titration with amethopterin (Fig. 10A) suggested 57% purity, i.e., 30% more enzyme. Calculations based on the inhibition of dihydrofolate reduction, in fact, yield higher values of purity.

Because of the variation in titration values (Figs. 9 and 10), selection of the data for calculating the molar concentration of the purified preparation was problematical and may be questioned. However, the selection was made primarily because the experimental conditions of folate reduction, rather than those of dihydrofolate reduction, more closely approximate those used by Werkheiser (29).

Several questions remain unanswered. Presently, the exact role of KCl is unknown. The rationale for the difference in titration values based on the variation in pH or temperature of the two enzymatic reductions may be erroneous. And, if inhibition by methasquin reflects interaction both at the active center and at another enzyme site, calculations based on titration with methasquin may be invalid. Conformational alteration dependent upon enzyme-methasquin interaction could increase the inhibitory activity of methasquin.

As a chemotherapeutic agent, methasquin may have greater value than amethopterin. Metabolites within the neoplastic cell which may interfere with the interaction of reductase and amethopterin may not affect the tenacious complex of enzyme and methasquin. These studies with the folate reductase of SF/A_k, in pointing to the multiple effects of the intracellular environment on drug action, yield this inference.

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